17β-Estradiol Increases Rat Cerebrovascular Prostacyclin Synthesis by Elevating Cyclooxygenase-1 and Prostacyclin Synthase

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Background and Purpose—It has been reported that estrogens modulate peripheral vascular synthesis of vasodilatory hormones, including prostacyclin. If this occurs in the cerebral circulation, it could have important consequences in the modulation of cerebral hemodynamic function and improvement of stroke outcome. We investigated the hypothesis that in vivo 17β-estradiol treatment of ovariectomized rats increases cerebrovascular prostacyclin production via elevation of the enzymes responsible for prostacyclin synthesis.

Methods—Cerebral blood vessels from 17β-estradiol–treated and nontreated ovariectomized rats were isolated and examined for prostacyclin synthesis by enzyme-linked immunosorbent assay or for protein levels of cyclooxygenase-1, prostacyclin-synthase, and cytosolic phospholipase A2 by immunoblot analysis.

Results—We report that chronic in vivo 17β-estradiol treatment significantly enhanced basal prostacyclin synthesis in rat cerebral blood vessels by 2.6-fold over control. 17β-Estradiol treatment also resulted in a 5.1-fold increase of cyclooxygenase-1 protein and a 6.7-fold increase of prostacyclin-synthase protein in the cerebral vasculature. There was no effect of estrogen on levels of cytosolic phospholipase A2.

Conclusions—Our findings suggest that estrogen influences the biosynthesis of prostacyclin, which may be important in the regulation of cerebral blood flow and thrombosis. This finding may shed light on the mechanisms that govern sex-based differences in cerebrovascular disease. (Stroke. 2002;33:600-605.)

Key Words: cerebral vessels ■ estrogens ■ prostacyclins ■ prostaglandin endoperoxide-synthase ■ rats

Epidemiological data indicate that the incidence of stroke is greater in men than in women throughout the world.1 In addition, premenopausal women have a lower stroke incidence than men; however, after menopause these differences dissipate.2-3 Since development of menopause is associated with increased stroke risk, estrogen has been implicated as a vasoprotective hormone. Though controversial, some clinical studies have shown that estrogen reduces cardiovascular morbidity and mortality in women receiving hormone replacement therapy.4-7 It is therefore of interest to characterize mechanisms by which estrogen may confer protection against cardiovascular diseases.

There is a rapidly accumulating body of evidence to suggest that estrogen mediates its cardioprotective effects via promotion of endothelial-derived vasodilator synthesis and release. Our laboratory has recently shown that chronic 17β-estradiol treatment of ovariectomized rats causes an increase of endothelial nitric oxide synthase in the cerebral microvasculature,8 as well as increasing nitric oxide synthase–mediated vasodilation in cerebral arteries.9 The ultimate consequence of endothelial nitric oxide synthase induction is an enhanced production of nitric oxide, a potent antithrombotic and vasodilatory substance.

Yet another vasoactive endothelial-derived hormone is prostacyclin (prostaglandin I2 [PGI2]). PGI2 levels have been shown to be increased by chronic 17β-estradiol exposure, especially in the aorta,10 pulmonary11 and uterine arteries,12 and umbilical veins.13 Moreover, fluctuating estrogen levels that occur during the menstrual cycle cause parallel changes in PGI2 synthesis in uterine blood vessels.14 Given that cyclooxygenase-dependent pathways are important in the regulation of cerebral blood flow,15 it is interesting that the effects of 17β-estradiol on PGI2 production in the cerebral vasculature have not previously been studied.

This study sought to answer 2 major questions: (1) Does chronic in vivo 17β-estradiol treatment enhance basal production of PGI2 in rat cerebral vessels? (2) Are the effects of 17β-estradiol on PGI2 levels mediated by treatment-induced changes in vascular levels of enzymes that are involved in PGI2 synthesis, namely, cytosolic phospholipase A2 (cPLA2), cyclooxygenase-1 (COX-1), and PGI2-synthase (PGI2-S)?

Materials and Methods

In Vivo 17β-Estradiol Treatment
Ovariectomized female rats (OVX group) and ovariectomized female rats treated with 17β-estradiol (OE group) were compared.
Ovariectomies were performed on 3-month-old Fischer 344 rats (Harlan Sprague-Dawley, Indianapolis, Ind) anesthetized by intraperitoneal injection of 46 mg/kg ketamine and 4.6 mg/kg xylazine. Hormone-treated rats received 17β-estradiol–filled silicone capsules that were subcutaneously implanted on the animal’s dorsum at time of ovariectomy. Implants were left in place for 4 weeks, after which animals were anesthetized by CO₂, and blood samples were obtained by cardiac puncture. Animals were then killed by decapitation, and serum levels of 17β-estradiol were measured by enzyme-linked immunosorbent assay (Diagnostic Products), and the action of 17β-estradiol was confirmed by measurement of dry uterus and body weights at time of death (Table). All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at the University of California at Irvine.

Cerebral Vessel Isolation

Two to 4 brains from each treatment group were pooled, homogenized with a loosely fitting Dounce tissue grinder in ice-cold 0.01 mol/L (pH 7.4) PBS, and centrifuged at 720g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was washed several times by resuspension in PBS followed by centrifugation at 720g for 5 minutes. The pellet was resuspended in PBS, then gently layered in a swinging bucket rotor at 4500g for 20 minutes at 4°C. The aqueous supernatant was discarded, and the layer containing parenchymal tissue was resuspended over dextran and centrifuged at 4500g for an additional 20 minutes. The pellets containing blood vessels were collected over a 50-μm nylon mesh and washed for several minutes with cold PBS. Blood vessels to be used for immunoblot analysis were frozen at −20°C until lysis, whereas those to be used for prostanoid production assays were used immediately. The isolated vessels, inspected by light microscopy, were a mixture of arteries, arterioles, veins, venules, capillaries, and associated perivascular elements.

Prostanoid Assay

Fresly isolated blood vessels from 4 OVX or 4 OE rats were finely resuspended in PBS, followed by centrifugation at 720g for 5 minutes. The pellets containing blood vessels were collected over a 50-μm nylon mesh and washed for several minutes with cold PBS. Blood vessels to be used for immunoblot analysis were frozen at −20°C until lysis, whereas those to be used for prostanoid production assays were used immediately. The isolated vessels, inspected by light microscopy, were a mixture of arteries, arterioles, veins, venules, capillaries, and associated perivascular elements.

Tissue Lysis and Protein Determination

Blood vessels were glass homogenized in lysis buffer (50 mmol/L β-glycerophosphate, 100 mmol/L NaVO₃, 2 mmol/L MgCl₂, 1 mmol/L EGTA, 0.5% Triton X-100, 1 mmol/L DL-dithiothreitol, 20 mmol/L pepstatin, 20 mmol/L leupeptin, 0.1 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride) and incubated on ice for 20 minutes. After homogenization, samples were centrifuged at 4500g for 10 minutes at 4°C. Supernatants were then collected, and protein content was determined by a modified Lowry assay. Samples were then separated by SDS-PAGE for immunoblot analysis.

Immunoblot Analysis

In all immunoblot experiments, equal protein amounts from OVX and OE preparations were run side by side on electrophoresis gels. Depending on the protein being detected, 30 to 50 μg of lysate protein was loaded onto 8% Tris-glycine gels and separated by SDS-PAGE. Then 50 μg of phosphor myristate acetate/lipopolysaccharide–induced or uninduced RAW 264.7 macrophage (Santa Cruz Biotechnology, Inc) or 25 μg of NIH/3T3 fibroblast (Santa Cruz Biotechnology, Inc) or human endothelial whole cell lysates (Transduction Laboratories) were loaded as positive controls for COX-2, COX-1, cPLA₂, and PGH₁-S immunoblots, respectively. Biotinylated molecular weight markers (Bio-Rad) were loaded as well. After electrophoretic separation, protein was transferred to nitrocellulose membranes (Amersham), which were subsequently incubated overnight at 4°C in blocking buffer containing 0.1% bovine serum albumin (BSA), 0.1% Tween-20 (T-PBS) and 6.5% nonfat dry milk. Membranes were then incubated with goat polyclonal anti–COX-2 (1:300; Santa Cruz Biotechnology, Inc), mouse polyclonal anti–COX-1 (1:350; Cayman Chemical), mouse polyclonal anti-cPLA₂ (1:100; Santa Cruz Biotechnology, Inc), or mouse polyclonal anti–PGH₁-S (1:100; Cayman Chemical) primary antibody in blocking buffer for 3 hours at room temperature and then washed 5×5 minutes in T-PBS at room temperature. Membranes were then incubated with either anti-goat (1:7500; Santa Cruz Biotechnology, Inc) or anti-mouse IgG–horse radish peroxidase (1:7500; Transduction Laboratories) in blocking buffer for 1 hour, after which they were washed 5×5 minutes in T-PBS, all at room temperature. The portions of membrane containing the biotinylated molecular weight markers were concomitantly incubated with streptavidin–horseradish peroxidase (1:6000; Sigma) for 1 hour, then washed 5×5 minutes in T-PBS. Membranes were incubated with electrochemiluminescence reagent (Amersham) for 1 minute and apposed to Hyperfilm (Amersham) for 30 seconds to 4 minutes depending on the antibody being used. The computer-based electrophoresis analysis program, UN-SCAN-IT (Silk Scientific), was used for densitometric quantitation of films. The density of the OE band is expressed as the mean fold increase over that measured for the OVX band for each protein detected.

Quantitative Analysis

Pixel density values from immunoblot films, 6-keto-PGF₁α, and TXB₂ levels from prostanoid assays, serum 17β-estradiol values, body weights, and uterine weights were compared between OVX and OE treatment conditions. Values are reported as mean ± SEM. Statistical differences in film band densities, serum 17β-estradiol, body weight, and uterine weight between treatment groups were determined by Student’s t test (GraphPad Prism 2.0 software). Differences in film band densities were assessed by paired analysis, whereas differences in serum 17β-estradiol, body weight, and uterine weight were determined by unpaired analysis. Differences in 6-keto-PGF₁α and TXB₂ levels at different time points were determined by 2-way ANOVA (SuperANOVA 1.11 software), followed by pairwise comparisons with Newman-Keuls post hoc analysis. For all comparisons, statistical significance was set at P≤0.05.

Results

In this study we used an in vivo rat model to determine the effects of chronic estrogen treatment on the cerebrovascular prostacyclin synthesis pathway. The Table compares mean
serum 17β-estradiol levels, dry uterine weights, and body weights between treatment groups. Serum taken from OE rats at time of euthanasia contained 17β-estradiol levels that were significantly elevated compared with that from OVX, as determined by radioimmunoassay (Table). These values approximate those found in normally cycling rats, cycling premenopausal women, and postmenopausal women on estrogen replacement therapy. This is a strong indication that our model achieved a sustained, chronic release of estradiol. Furthermore, released estradiol had bioactivity, as evident in the hypertrophied uteri from OE rats versus the atrophied uteri from OVX rats (Table). Estrogen treatment also resulted in significantly lower body weights compared with OVX, which is in support of human studies.

To assess the effects of chronic estrogen treatment on basal PGF₁₀ synthesis, we quantitatively measured 6-keto-PGF₁₀ levels in the tissue incubation medium by ELISA analysis. Figure 1 illustrates 6-keto-PGF₁₀ levels above background, produced by cerebral blood vessels taken from OVX and OE rats. At 15, 30, and 60 minutes, cerebral vessels from OE rats displayed significantly higher (P<0.05) basal synthesis of 6-keto-PGF₁₀ compared with vessels from OVX animals. At time points before 15 minutes there was a trend toward increased PGF₁₀ synthesis; however, statistically significant differences were not achieved until the 15-minute time point. Furthermore, we examined vascular production of TXA₂, measured as TXB₂, to determine whether the effects of estrogen were specific to the prostacyclin pathway or whether synthesis of prostanoids in general was upregulated. Cerebral blood vessels synthesized TXA₂ over the 1-hour incubation period, albeit at levels much lower than PGF₁₀ (Figure 1). No significant differences in TXA₂ levels were detected between OVX and OE at any of the 5 time points measured; however, given the variability of these data, statistical power for these comparisons was low (for example, at 15 minutes, OE=25±9 versus OVX=13±3 pg TXB₂ per microgram protein; at 30 minutes, OE=49±19 versus OVX=26±10 pg TXB₂ per microgram protein; n=5).

Immunoblot studies were performed to examine the effects of chronic estrogen treatment on the levels of enzymes involved in the cerebrovascular PGF₁₀ synthesis cascade. Blots probed with polyclonal antibodies directed toward cPLA₂ detected single bands migrating at approximately 110 kDa (Figure 2). This corresponds to the molecular weight of this arachidonic acid–liberating enzyme as well as to the band visualized in the positive control. No bands were detected when the primary antibody was omitted, suggesting a specific and selective primary antibody–protein interaction. Densitometric analysis of autoradiographic films revealed no difference in cPLA₂ protein levels between cerebral blood vessels from OVX and OE rats (P=0.59; n=4) (Figure 2). These differences are expressed in Figure 2 as a 0.99±0.12-fold change in cPLA₂ in OE versus OVX rats.

Blots probed with anti–COX-1 polyclonal antibodies detected bands at 70 kDa corresponding to the molecular weight of the COX-1 protein, as well as to the positive control (Figure 3). In contrast, no COX-2 was detected in cerebral blood vessel lysates (limit of detection was 20 pg COX-2 protein per microgram vessel lysate protein), although a strong positive control band was detected (data not shown). Chronic 17β-estradiol treatment resulted in a significant elevation (P<0.05) of cerebrovascular COX-1 levels compared with OVX. These differences are expressed in Figure 3 as a 5.1±1.2-fold increase in COX-1 in OE versus OVX rats.

There also was a significant effect (P<0.05) of 17β-estradiol treatment on PGF₁₀-S levels in rat cerebral blood vessels (Figure 4). Bands detected by anti–PGF₁₀-S polyclonal antibodies at 56 kDa were increased in OE versus OVX rats by 6.7±1.2-fold (Figure 4). Immunoblot experiments with omission of the primary antibodies resulted in loss of band detection for both COX-1 and PGF₁₀-S. Furthermore, as a loading control we probed with an anti-cPLA₂ polyclonal antibody. Bands were detected at 110 kDa, which corresponds to the molecular weight of the cPLA₂ protein. A representative blot is shown. Densitometric data are illustrated as fold increase compared with OVX group. Values represent mean±SEM (n=4). Chronic in vivo treatment of OVX rats with 17β-estradiol did not alter cPLA₂ levels in the cerebral vasculature.
estrogen plays a key role in the modulation of cerebral vascular contractile function by specifically promoting the elaboration of a potent vasodilatory, antithrombotic, and COX-1-derived substance, namely, PGI₂. This regulation may represent one mechanism by which estrogens confer premenopausal women with heightened protection against cardiovascular diseases, including stroke.5

The association of estrogen with elevated PGI₂ has been documented for the general circulation and peripheral vasculature. Studies in both animals and humans indicate that during pregnancy there is a considerable elevation of PGI₂ metabolite levels in both blood and urine.19–21 In contrast, after menopause there is a decline in urine PGI₂ metabolite levels22; however, postmenopausal women receiving estrogen replacement therapy show increased urinary excretion of PGI₂.23 The source of these changes is likely to be vascular tissue because the endothelium is the body’s largest source of PGI₂.

These reports of the effects of estrogen on levels of prostacyclin are corroborated by observations that estrogen can directly stimulate PGI₂ synthesis in noncerebral vascular cells in vitro. For example, tissue culture studies have demonstrated that 17β-estradiol exposure stimulates PGI₂ biosynthesis in rat aortic smooth muscle cells24 and in rat aortic,10 human umbilical vein,13 and ovine pulmonary artery endothelial cells.11 Here we have extended these findings to the brain vasculature by showing for the first time that intact female rat cerebral blood vessels exposed to 17β-estradiol for 1 month are capable of producing higher basal levels of PGI₂ than those of their estrogen-deficient counterparts. A number of studies indicate that endothelial cells are the primary source of vascular PGI₂;25; however, possible contributions from other cell types in the intact cerebral vessels, eg, smooth muscle, pericytes, adventitial nerves, and astrocytic endfeet,26 cannot be ruled out. Another notable difference between our in vivo paradigm of estrogen treatment and cell culture studies is that intact organisms are capable of hepatic, as well as extrahepatic, metabolism of drugs and hormones. Thus, it is possible that, in vivo, metabolites of 17β-estradiol may also exert a PGI₂-stimulatory role in the vasculature. In fact, it has recently been shown that estrone, 2-methoxyestrone, 2-methoxyestradiol, and 16α-hydroxyestrone, all metabolites of 17β-estradiol, are potent promoters of PGI₂ synthesis in cultured human umbilical vein endothelial cells.27 Therefore, it is conceivable that these and other 17β-estradiol metabolites may subserve a similar role in the living animal.

When we consider that gonadal steroid hormones bind intracellular receptors, translocate to the nucleus as a hormone-receptor complex, and interact with estrogen response elements to induce gene transcription, it seems likely that estrogen increases expression of genes whose products are involved in the prostacyclin synthesis pathway. Indeed, a particular study of ovine pregnancy demonstrated that, during the last trimester when serum estrogens are dramatically elevated, increased uterine arterial PGI₂ is largely attributable to increased expression of COX-1 mRNA and protein in the endothelium, with smaller increases occurring in the smooth muscle.12 This same group also demonstrated that COX-1 mRNA and protein are upregulated during the follicular phase (high estrogen) of the ovine menstrual cycle compared with the
luteal phase (low estrogen). In another study treatment of fetal ovine pulmonary artery endothelial cells with 17β-estradiol in vitro increased COX-1 mRNA and protein with a concomitant elevation of both basal and stimulated PGI2 synthesis. This was shown to be due to increased transcription and translation and not to changes in stability of mRNA or protein. Furthermore, these modulatory effects of 17β-estradiol were inhibited by the estrogen receptor antagonist ICI 182,780, suggesting that receptor-mediated mechanisms are involved.

In studies of human umbilical vein endothelial cells, enhancement of PGI2 synthesis by 17β-estradiol was shown to be sensitive to the partial agonist tamoxifen, further supporting a role for the classic estrogen receptor.

In the present study in vivo 17β-estradiol treatment increased COX-1 and PGI2-S in rat cerebral blood vessels, consequently elevating PGI2 production. Since we did not administer an estrogen receptor antagonist in conjunction with 17β-estradiol, we cannot definitively state that 17β-estradiol is acting through receptor-mediated mechanisms to increase expression of COX-1 and PGI2-S gene products and thus increase PGI2 production. However, findings made by our group and others provide evidence that this is the likely mechanism mediating the observations reported in this study. First, the half-palindromic GGTC sequence, known to act as an estrogen response element, has been identified in the 5'-flanking region of both the COX-1 and PGI2-S genes. However, it is possible that estrogen can also regulate transcriptional activity by interacting with other types of transcription factors important in modulating the expression of COX-1 and PGI2-S. Second, recent work in our laboratory has identified estrogen receptor α protein in cerebral blood vessel preparations from female rats (Stirone C, BS, et al, unpublished data, 2001). Third, we have shown that in vivo 17β-estradiol treatment in mice augments COX-mediated changes in mouse cerebral artery diameter measured in vitro. Furthermore, these differences are abolished in estrogen receptor α knockout animals (αERKO). Finally, whereas in vivo 17β-estradiol treatment of wild-type OVX mice increases COX-1 in cerebral blood vessels, there was no effect of estrogen on COX-1 in αERKO mice. One can therefore speculate that the effects of estrogen that we have demonstrated on COX-1 and PGI2-S are mediated by receptor-induced alterations in gene transcription. Additional studies combining quantitative analysis of cerebrovascular mRNA levels with examination of the effects of estrogen receptor antagonists would be necessary to more fully define the mechanism by which estrogen regulates prostacyclin production in the cerebral circulation.

One end point unaffected by chronic 17β-estradiol treatment was cPLA2, the enzyme responsible for catalyzing the release of arachidonic acid from membrane phospholipids, and thus the initial step in the prostaglandin synthesis cascade. This is in contrast to prior reports showing that 17β-estradiol promotes increases in cPLA2 protein, albeit in the quail oviduct. On the other hand, in support of our findings, it was reported that prolonged exposure of ovariectomized sheep to 17β-estradiol had no effect on cPLA2 protein levels in the uterine artery. Since cPLA2 is involved in many cellular processes, including signaling pathways and prostaglandin and leukotriene synthesis, it is not surprising that estrogen does not specifically regulate its production in vascular tissue.

A general caveat regarding the interpretation of treatment-induced molecular alterations in animal tissues stems from whether these changes correlate with real physiological and pathophysiologic consequences in the intact organism. Although we report increases in basal PGI2 synthesis and cerebrovascular COX-1 and PGI2-S protein in response to chronic 17β-estradiol treatment, we did not determine whether these effects result in changes in cerebral blood flow. However, there is a growing body of evidence from both humans and animals to suggest that gonadal hormones do indeed affect responsiveness of the cerebral circulation via changes in COX-dependent pathways. Doppler ultrasonography has been used to evaluate hemodynamic parameters in the cerebral microcirculation of pregnant, premenopausal, and hypoestrogenic postmenopausal women. These studies showed that estrogen status does indeed affect cerebrovascular reactivity. Estrogen treatment of castrated male rats has also been shown to increase endothelium-dependent dilation of isolated, pressurized middle cerebral arteries. In a parallel study, chronic administration of 17β-estradiol to OVX female mice increased middle cerebral arterial diameter by enhancing COX-dependent mechanisms. Both resting and agonist-stimulated changes in cerebral blood flow appear to be attenuated by COX-1 inhibitors in humans and animals. Furthermore, COX-1 null mice exhibit diminished resting cerebral blood flow, as well as attenuated vasodilator responses to bradycin and A23187. Thus, there is good evidence for the importance of COX-dependent pathways in cerebral hemodynamic regulation. It is likely that upregulation of PGI2 synthesis by estrogen has significant physiological ramifications in humans.

The correlation of our present biochemical observations with physiology data previously provided fits well into the current framework of knowledge regarding the beneficial effects of 17β-estradiol therapy on stroke outcome. 17β-Estradiol administration in gonadectomized male and female rats reduces caudoputamen and cortical infarct volume sizes after reversible middle cerebral artery occlusion. Although it has been suggested these effects are due to estrogen-mediated neuronal protection against ischemic damage, work from our group indicates that preservation of blood flow, by upregulation of COX- and endothelial nitric oxide synthase–dependent pathways, deserves consideration as a mechanism by which 17β-estradiol improves stroke outcome. In support of this, chronic 17β-estradiol treatment followed by global cerebral ischemia resulted in increased cerebral blood flow during the ischemic period in addition to a reduction in postischemic hyperemia. Resistance to ischemic insults resulting from estrogen-induced vascular changes might explain the myriad of reports indicating that estrogen makes women less likely to experience, and die of, a stroke than men.

In summary, the data provided in this report are the first to show that chronic 17β-estradiol treatment results in enhanced cerebrovascular basal PGI2 production, likely due to treatment-induced increases in the enzymes associated with PGI2 synthesis (COX-1 and PGI2-S). Although we have not characterized the exact cellular location of these hormonal effects because of the limitations of using whole tissue...
preparations, immunohistochemical analysis might be useful in providing an answer to this question. Nevertheless, these findings provide insight into the vascular mechanisms by which estrogens modulate cerebral vascular function.

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References

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